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# Nanofluidic Fluorescence Microscopy (NFM) for real-time monitoring of protein binding kinetics and affinity studies

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ABSTRACT

Kinetic monitoring of protein interactions offers insights to their corresponding functions in cellular processes. Surface plasmon resonance (SPR) is the current standard tool used for label-free kinetic assays; however, costly and sophisticated setups are required, decreasing its accessibility to research laboratories. We present a cost-effective nanofluidic-based immunosensor for low-noise real-time kinetic measurement of fluorescent-labeled protein binding. With the combination of fluorescence microscopy and reversed buffer flow operation, association and dissociation kinetics can be accessed in one single experiment without extra buffer loading step, which results in a simplified operation and reduced time of analysis compared to typical microfluidic immunoassays. Kinetic constants of two representative protein-ligand binding pairs (streptavidin/biotin; IgG/anti-IgG) were quantified. The good agreement of extracted rate constants with literature values and analogous SPR measurements indicates that this approach is applicable to study protein interactions of medium- and high-affinities with a limit of detection down to 1 pM, regardless of the analyte size.



## 1. Introduction

Analysis of protein interactions provides a fundamental understanding of their physiological functions at the cellular scale in virtually all chemical and biological processes. These studies offer essential information for the development of improved diagnostic tools and new therapeutic treatments against diseases (Ideker and Sharan, 2008). Over the last few decades, affinity based biosensors, exploiting the interaction between a free target analyte and an immobilized receptor on a solid surface, have been a key solution in characterizing biospecific interactions in vitro. This method reveals the affinity and kinetic information of various binding events particularly protein-ligands and nucleic acids (Malmqvist and Karlsson, 1997; Nguyen et al., 2007). Affinity biosensor implemented on solid-state device that couple immunochemical reactions to a transducer is termed as immunosensor. Immunosensors can be divided into different categories depending on the detection principles, for example, optical, electrochemical, microgravimetric and thermometric (Luppa et al., 2001) schemes. Optical based immunosensors are the most widely used in clinical, food and environmental applications due to their performance characteristics.

Among optical based immunosensors, surface plasmon resonance (SPR) is currently the standard commercialized technology routinely used in the field of pharmaceutical and life sciences, offering a real-time detection of broad range of biomolecular interactions without labeling requirement (Myszka and Rich, 2000). Practically, measuring the interactions of low-molecular weight analytes such as hormones, antibiotics, and chemical drug-fragments with SPR instrument is a key limitation in terms of sensitivity. Furthermore, if the reaction is too fast, with a high association rate, the obtained kinetic information is not reliable due to the strong effect of mass transfer (Nguyen et al., 2007). More importantly, high-cost dedicated sensor surfaces and integration of optical components are required, which in turn increase the overall assay costs and complicate the instrument setup.

Traditional fluorescence stopped-flow has been a method of choice for most biologists to study millisecond kinetic reactions of various biomolecular interactions in solution, for example, protein-DNA interactions (Esadze and Iwahara, 2014), ligand binding (Olsen et al., 1992) and enzyme reactions (Zhang et al., 2004).

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Total-internal-reflection fluorescence (TIRF) is a powerful analytical tool in visualizing real-time reversible interactions of fluorescent species on the surface with single-fluorophore sensitivity and minimized background fluorescence (Lakowicz, 2006; Schneckenburger, 2005). TIRF has been employed in combination with fluorescence polarization (FP) and fluorescence correlation spectroscopy (FCS) to determine affinities and reveal binding kinetics of fluorescently labeled IgG with mouse receptor Fcgammaril and human estrogen receptors with their specific ligands (Kwok and Cheung, 2010; Lieto et al., 2003), with nanoscale spatial resolution. Other fluorescence based techniques enabling real-time measurement of biomolecular recognition events include fluorescence after photo bleaching (FRAP) (Lele and Ingber, 2006) and Förster resonance energy transfer (FRET) (Li et al., 2007). On top of everything, Episentec has newly developed an add-on tool, the label-enhanced SPR technology, breaking the performance barriers of conventional SPR. This new technology aims at enhancing sensitivity and selectivity together with capability to analyze small molecules and fast kinetic reactions by using developed Episentec™ dye labels (Granqvist et al., 2013). These aforementioned examples have clearly proved that label-based techniques are still up-to-date and are readily applicable for investigating binding kinetics of ligand-receptor interactions in complement with label-free techniques to bring on biologically meaningful information.

In parallel, we have lately witnessed remarkable progress of novel micro- and nano-fluidic devices, allowing single molecule analysis (Dittrich and Manz, 2005; Yeh et al., 2012), cell sorting (Mazutis et al., 2013; Wang et al., 2005), DNA separation (Ashton et al., 2003; Fu et al., 2007), and fast multiplexed protein detection (Didar et al., 2012; Mitsakakis and Gizeli, 2011). The combination of nanofluidics with advanced biosensor technology paves new roads for point-of-care clinical diagnostics particularly owing to their capability to integrate such devices into lab-on-a-chip as the dimensions scale down. Nanofluidic-based biosensors predominately reduce the consumption of costly biological reagents with enhanced speed of analysis thanks to the shortened diffusion distance between immobilized probe molecules and flowing analytes in highly confined channels. First attempts to use biofunctionalized nanochannel for detection of streptavidin molecule using label-free impedance measurement in liquid induced flow has been reported (Karnik et al., 2005; Schoch et al., 2007). Though enhanced surface binding reactions have been demonstrated using pressure-driven flow over diffusion-limited reactions, long response time of 1–2 h were required due to the lack of localized selective surface modifications (Schoch et al., 2007).

To address the issue of localized and selective surface modifications in nanochannels, we have developed a strategy using pre-functionalized nanofluidic channels with biocompatible encapsulation technique for DNA and protein detection (Leïchlé et al., 2012). We have shown that by confining the reaction volume into sub-μm thick channels, fluorescence conjugated biomolecules can be detected in real-time without cumbersome washing steps using a conventional fluorescence microscope (Leïchlé and Chou, 2015). This unique feature is specific to nanometer scale channels because the sample volume reduction during observation leads to negligible levels of fluorescence background, thus allowing us to directly probe the sensing surface without the need of using complicated and expensive setups, such as SPR, TIRFM, or quartz crystal microbalance (QCM). Moreover, the drastic reduction of diffusion length permits to operate in a reaction-limited regime with optimized target capture efficiency. Hence, all target molecules injected in the device are analyzed and because the typical sample volume is in the pL range, i.e. 4 orders of magnitude smaller than the sample volume of conventional microfluidic biosensor formats (Darain et al., 2009), this platform is especially suitable to the analysis of ultra-small samples, e.g. the content of few cells.

Here, we further demonstrate that this cost-effective sensing platform relying on the combinational use of biofunctional nanoslits and fluorescence detection, can achieve real-time monitoring of protein binding kinetics in physiological liquid environment with kinetic constant extraction, a platform we called Nanofluidic Fluorescence Microscopy (NFM). Fluorescence microscopy offers the possibility to carry out spatially resolved measurements where each camera pixel represents an independent measurement of the reaction kinetics. Hence, in addition to the high sensitivity with no analyte mass dependence inherent to this detection technique, large sampling area over a number of pixels ensures reduced statistical errors. On the other hand, because the fluorescent background level is proportional to the channel height, sub-micrometer channels offer signal to noise ratio of at least 100 on a large scale of dissociation constant $K_D$, in theory from the pM to the sub-μM range, that concerns most molecules of interests. Here, two representative protein-receptor pairs of different affinities, streptavidin/biotin (high affinity) and mouse IgG/anti-mouse IgG (medium affinity), were chosen to demonstrate the capability of our devices to determine kinetic parameters. Binding kinetics of streptavidin/biotin interaction was investigated in our study since monitoring this high affinity recognition with the standard SPR method has not yet been addressed. IgG/anti-IgG pair was chosen as a generic candidate model for other types of protein-ligand interactions.

Molecular interactions within biofunctionalized nanoslits were visualized by classical fluorescence microscope under pressure-driven flow (Fig. 1a). The results presented here show that our device was capable of generating full kinetic sensorgram including both association and dissociation phases in one single-experiment with a single injection via reversed buffer flow operation, which is another golden feature of our device (Fig. 1b). Indeed, because we operate in full target capture, all upcoming molecules injected onto the sensor are consumed by the reaction and a diffusing layer (depleted layer) forms at the sensor site. This depleted layer constitutes a clear boundary between regions where the concentration of analyte drops from maximum to zero. Thus, the liquid downstream the sensor is analyte free and the dissociation study can be simply implemented after completion of the association phase by reserving the fluid flow within the nanochannel instead of injecting new buffer in the inlet, which results in a simplified operating protocol and reduced time of analysis. The extraction of binding constants was conducted using a finite element model, an analytical method, and a commercial software package to demonstrate that our kinetic data could steadily be applied to various fitting approaches. To validate the performance of our proposed device for kinetic constant determination, the extracted on/off rates were compared with the ones from literatures and with analogous SPR measurements.

## 2. Material and methods

### 2.1. Surface chemistry

For selective immobilization of the bio-receptors on the surface inside nanoslits, gold-thiol chemistry was utilized via self-assemble monolayer (SAM) formation (Fig. 2). Gold surface was chosen for the local surface immobilization of bioreceptor probes in order to work with surfaces similar to the ones used in common SPR measurements. In this work, SAMs are composed of two thiol species, (1) hydroxyl-terminated thiol or a spacer and (2) biotinylated thiol used for further modification via biotin-streptavidin linkage. Optimization of the mixing ratio between these two

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